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Susceptibility to Atherosclerosis in Aortas and Coronary Arteries of Swine with von Willebrand's Disease

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16. ABSTRACT

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JOURNAL ARTICLE

Susceptibility to Atherosclerosis in Aortas and Coronary Arteries of Swine With von Willebrand's Disease

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The development of coronary and aortic atherosclerosis was determined after balloon catheter injury of coronary arteries and administration of an atherogenic diet in normal pigs and pigs that were homozygous and heterozygous for von Willebrand's disease. Coronary atherosclerosis developed to a similar degree in all three phenotypic groups. The mean intimal thickness at the site of maximal thickness in ballooned vessels was .51 mm in the normal pigs, .67 mm in carrier pigs, and .55 mm in bleeder pigs. The intimal thickness of nonballooned vessels was .28 mm in normal pigs, .28 mm in carrier pigs, and .35 mm in bleeder pigs. Fibrous lesions of atherosclerosis covered an average of 3.88% of the aorta in normal pigs, 2.83% in carrier pigs, and 2.37% in bleeder pigs. The difference between the aortic lesions of normal animals and bleeders was significant ($P < .05$). Absence of von Willebrand factor was associated with limited resistance to atherosclerosis in the aortas of experimental pigs but did not affect the development of atherosclerosis in either ballooned or nonballooned coronary arteries. These findings suggest, first, that von Willebrand factor function is not essential to the development of the atherosclerotic lesion in this model and, second, that the role of the von Willebrand factor in the development of atherosclerosis is complicated and appears to involve interaction with variables not yet defined. (Am J Pathol 1981, 102:137–145)

MANY INVESTIGATORS have demonstrated an intimal proliferative response to endothelial injury in experimental animals.[1-5] Because of such observations, these investigators and others have hypothesized that vascular injury is an initiating event in the evolution of atherosclerosis. This hypothesis was given new impetus when Ross et al showed that platelets could release a factor that promoted smooth muscle cell growth in culture systems.[6]

The description of a platelet-derived growth factor stimulated the proposal that the early lesion of atherosclerosis results because an endothelial injury is followed by platelet deposition and release of a mitogenic factor.[7] Several lines of evidence support this notion by showing that depression of platelet function has a protective influence on the development of atherosclerosis in experimental animals. Among these are the studies by Friedman et al[8] that showed depression of smooth muscle proliferation in ballooned rabbit aortas after administration of an antiplatelet antibody. Second, there are reports from Harker et al[9] and from Pick et al,[10] who studied monkeys, and from Fuster et al,[11] who studied swine, showing some resistance to diet-induced atherosclerosis in animals treated with aspirin and other medications that inhibit platelet function. The injury-platelet response hypothesis was further supported by the observations of Fuster et al, who showed that swine with von Willebrand's disease are resistant to development of diet-induced aortic atherosclerosis.[12] Pigs homozygous for von Willebrand's disease have prolonged bleeding times[13] and a deficiency of a plasma factor (von Willebrand factor) thought to support normal platelet adherence to injured vascular tissue.[14]

We designed this study to determine whether the protection from atherosclerosis enjoyed by pigs with von Willebrand's disease would be demonstrable even if the vascular endothelium were completely denuded by a balloon catheter. Failure of animals with

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von Willebrand disease to develop intimal thickening after balloon injury would suggest an important, independent role of the von Willebrand factor in the processes of vessel repair and atherogenesis. On the other hand, development of atherosclerosis in bleeder animals in response to balloon injury and the atherogenic diet would imply that the von Willebrand factor functions as a covariable in these processes. The pigs with von Willebrand's disease would become, if the latter case were shown, an important model for isolating the effects of the von Willebrand factor in relation to the other variables.

Balloon injury was administered to coronary arteries, and the aortas were used as nonballooned controls. Our results show that while bleeder pigs develop significantly less aortic atherosclerosis than do normal pigs, the two groups of animals develop atherosclerosis in coronary arteries at similar rates.

Materials and Methods

Swine were from the inbred colony of the von Willebrand's disease strain in Chapel Hill. The original breeding stock was described by Hogan, et al in 1941.[15] This strain of swine has a bleeding diathesis similar to von Willebrand's disease of humans. The characteristics of the bleeding disease in the homozygote include a long bleeding time, a low level of plasma factor VIII (antihemophilic factor), a marked and prolonged response of plasma factor VIII levels to plasma infusions, and undetectable levels of von Willebrand factor.[14]

Accelerated atherosclerosis was induced by the combined use of balloon endothelial injury of the coronary arteries and administration of an atherogenic diet. Pigs weighing 10–33 kg were anesthetized with intramuscular ketamine and halothane. A No. 4 French Swan-Ganz catheter was manipulated into one or two of the three major coronary arteries via a carotid arteriotomy.[2] The most frequently entered coronary artery was the left anterior descending. When the balloon tip was in the artery, the balloon was inflated to the point of firmness, then pulled quickly to the aorta. Each artery entered was ballooned two to four times. During the procedure, the pressure at the tip of the Swan-Ganz catheter and the electrocardiogram were monitored. After the ballooning procedures were completed, the animals were transported to individual pens, where they were housed for the remainder of the experimental period.

During the following 4 months, the pigs were fed a diet containing 2% cholesterol, 0.75% sodium cholate, and 20% beef tallow. Before the ballooning procedure, and at monthly intervals after the ballooning, an electrocardiogram, hematocrit, platelet count, and cholesterol levels were measured on each animal.

At the end of a 4-month period the animals were sacrificed by injection of a concentrated solution of potassium chloride. Two pigs of each phenotype were sacrificed by pressure perfusion with 4% paraformaldehyde after being anesthetized. All aortas were opened along the anterior surface and pinned to a corkboard for examination and photography. Samples of fresh arterial wall were sectioned in a cryostat, then stained with oil red 0 or osmium for demonstration of lipid content. The remainder of the aortic tissue was fixed in 10% buffered formalin. At a later time the aortas were stained with Sudan IV. A template method was used to measure the extent of the atherosclerotic lesion as a function of the area of the total aortic luminal surface. The template tracings were made on transparent X-ray films that were laid over the pinned-open aortas. The grading procedure was done by three observers who did not know the phenotype of the pigs from which the aortas were taken. The results of these determinations were averaged.

The hearts were examined grossly and photographed. The coronary arteries were then examined by postmortem angiography by the method of Suberman et al.[16] After the X-ray procedure was completed, the coronary arteries and aorta were dissected from the heart. Most of the hearts were examined grossly after being sliced into multiple transverse sections. Some were opened along the border of the anterior interventricular septum and the anterior free wall so that the left ventricle could be laid out for examination. Sections were taken from selected areas of myocardium for light microscopy.

The coronary arteries were cut in 5-mm sections. The step sections were photographed grossly on end and embedded in paraffin for histologic examination. The sections were graded by two observers for the degree of intimal thickening. A micrometer was used to measure the intimal and medial thickness at the point of maximal thickness in each vessel.

Laboratory Analyses

The von Willebrand factor (platelet-aggregating factor, PAF) was measured by the macroscopic aggregating test with the use of formalin-fixed human platelets.[14,17] Factor VIII coagulant activity (antihemophilic factor, AHF) was measured with the partial thromboplastin time assay with a canine hemophilic plasma as substrate.[18] Bleeding time was measured by the use of the ear and immersion in saline as de-

Table 1—Characteristics of Experimental Pigs

No. Pigs	Mean PAF (%)	Months on diet	Mean weight (kg)	Mean hematocrit (vol %)	Mean platelet count (× 10^3/cu mm)	Mean serum Cholesterol (mg/dl)
Normal pigs						
6	123 ± 21.3	0	22 ± 6.6	40 ± 1.7	572 ± 68	113 ± 23
6		2	42 ± 10.0	33 ± 3.4	592 ± 153	747 ± 151*
6		4.2	58 ± 5.8	33 ± 3.5	542 ± 181	629 ± 186
Carrier pigs						
8	66 ± 11.5	0	21 ± 5.9	36 ± 4.4‡	689 ± 94†	117 ± 60
8		2	36 ± 9.0	32 ± 2.4	601 ± 126	759 ± 205
7		4.3	62 ± 11.5	32 ± 2.8	538 ± 131†	755 ± 280
Bleeder pigs						
7	< 1	0	19 ± 3.0	35 ± 6.3	671 ± 199†	135 ± 40
7		2	36 ± 5.6	32 ± 4.2	636 ± 135†	540 ± 218
7		4.2	50 ± 9.5	30 ± 6.5	561 ± 225	635 ± 265

* Five pigs only.
† Six pigs only.
‡ Seven pigs only.

scribed by Mertz.[13] Serum cholesterol values were determined by an automated technique.

Results

Table 1 shows the average PAF levels, body weights, hematocrits, platelet counts, and serum cholesterol levels of the experimental animals. Initial weights of the animals in each phenotype varied from 10 to 33 kg. The mean initial weights in each group were not significantly different, however. Terminal weights, on the other hand, tended to be lower among the bleeder animals.

The hematocrits were generally low among all of the test animals, with bleeder animals showing a lower level than that of the other two phenotypic groups. Most bleeder animals experienced some nasal mucosal hemorrhage during the 4-month experimental period. None were transfused. One bleeder animal died from gastrointestinal hemorrhage after 2 months on the atherogenic diet. Data from that pig were excluded from this report. Platelet counts fell in all of the study groups during the experiment. All counts were within normal limits, however, and there was no demonstrable difference between phenotypic groups. Mean serum cholesterol levels were similar in the three phenotypic groups, although the variability among individual pigs was considerable.

Coronary Atherosclerosis

All of the experimental pigs showed some degree of coronary and aortic atherosclerosis. Coronary arteries that were balloon-injured were usually more severely diseased than those that were not ballooned (Figures 1 and 2).

Table 2—Extent of Sudanophilia and Distal Fibrous Plaque Lesions of the Aorta in Normal Pigs and Pigs Heterozygous (Carrier) and Homozygous (Bleeder) for von Willebrand's Disease

Normal			Carrier			Bleeder		
Pig No.	Sudanophilia (% total area)	Distal fibrous plaque (% total area)	Pig No.	Sudanophilia (% total area)	Distal fibrous plaque (% total area)	Pig No.	Sudanophilia (% total area)	Distal fibrous plaque (% total area)
1	53.0	2.8	7	22.5	2.9	13	21.3	1.1
2	54.5	6.7	8	28.9	2.3	14	33.0	1.3
3	54.6	2.7	9	57.8	1.5	15	53.6	3.5
4	57.6	2.7	10	48.2	1.5	16	28.7	1.5
5	37.1	2.9	11	59.4	3.7	17	55.6	3.1
6	63.7	5.5	12	66.3	5.1	18	59.2	3.3
						19	68.1	2.8
Mean	53.4	3.88		47.18*	2.83*		45.64*	2.37†
SD	± 8.86	± 1.76		± 17.73	± 1.40		± 17.75	± 1.03

* Not statistically different from the mean in normal pigs.
† $P < .05$, in comparison with normal pigs.

Gross narrowing of the vascular lumen was noted in at least one coronary artery in 3 of the 6 normal pigs, in 7 of 8 carrier pigs, and in 4 of the 7 bleeders. In the 6 normal pigs, 2 of 11 ballooned arteries and 2 of 7 nonballooned vessels were grossly narrowed. Fourteen arteries were ballooned in the 8 carrier pigs. Nine of these were narrowed grossly. None of the 10 nonballooned arteries in carrier pigs were narrowed. Among the 7 bleeder pigs, 5 of 13 ballooned arteries were narrowed, while 2 of 8 nonballooned arteries were grossly narrowed. The only lesion associated with gross narrowing was a fibroproliferative plaque involving part of the circumference of the vessel and oriented along the long axis of the vessel (Figure 1). On cross-section, these lesions had a thick fibrous and cellular luminal cap covering a core filled with lipid-laden cells and free lipid. The boundary between intima and media in many of these vessels was obscured because of disruption and reduplication of the internal elastic lamina. Most of these lesions contained focal areas of calcification. There was no detectable difference in the degree of calcification of the lesions among the phenotypic groups. Similarly, we detected no association between the degree of calcification and the size or composition of the lesions. Many areas that were not involved with the fibroproliferative lesion were entirely normal in histologic appearance. However, some areas showed a mild degree of intimal thickening with accumulation of foam cells. These areas were especially prominent at points of bifurcation of the arteries. The foam cell thickening was not visible by gross examination, nor were

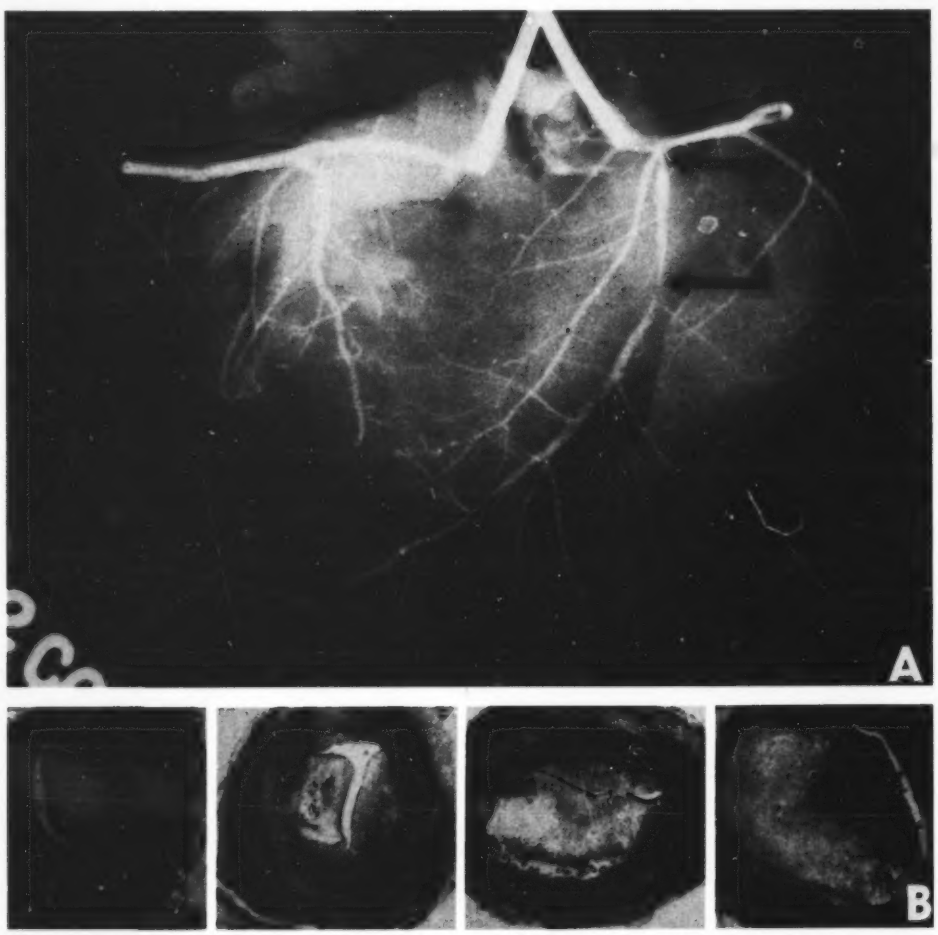

Figure 1A—Postmortem angiogram from a normal pig showing narrowing of two segments of the left anterior descending coronary artery. Both the left anterior descending, and the circumflex coronary arteries were balloon-injured. **B**—Sequential sections from an area corresponding with the proximal left anterior descending coronary artery shown above. (H&E, × 10)

these areas associated with narrowing that could be shown by postmortem angiography. The mild degree of thickening associated with the foam cell accumulations was quantitatively similar in all three phenotypes. We were unable to demonstrate a difference in the degree of coronary intimal thickness between the three phenotypic groups (Figure 2).

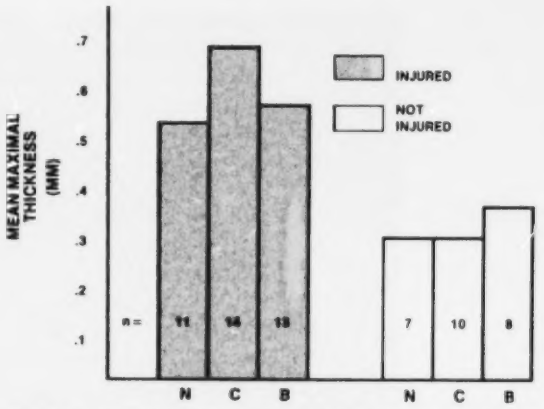

Figure 2—Maximal thickness of the intima (mm) at the site of greatest thickness. Five-millimeter step sections of coronary artery were examined. Balloon-injured vessels are grouped on the left for comparison with nonballooned vessels on the right. *N*, normal or wild type; *C*, carrier or heterozygote; *B*, bleeder or homozygote for von Willebrand's disease. Differences between phenotypes in the ballooned and nonballooned groups are not significant. Differences between ballooned and nonballooned groups are significant ($P < 0.025$).

Aortic Lesions

The aortic lesions that developed in the pigs, regardless of phenotype, were grossly and microscopically similar to the three types of lesions described in other studies of porcine atherosclerosis[19] (Table 2). First, there was an extensive degree of sudanophilia in all of the pigs (50% of the aortic surface on average) (Figure 3). The sudanophilia was most prominent in the proximal section of each of the aortas. There was no significant difference in the degree of aortic involvement with sudanophilia among the three phenotypes.

The second lesion was a raised fatty lesion that covered a highly variable area (Figure 4). These lesions were very poorly demarcated from the surrounding flat intima and were generally of somewhat greater than 100 μ in thickness before they could be readily detected by gross examination. They were composed of smooth muscle cells and various amounts of lipid. Many of the lesions contained prominent calcification. These lesions were by no means confined to the intima of the vessel and frequently involved the luminal half of the media. It was difficult to quantify the relative extent of the lesions

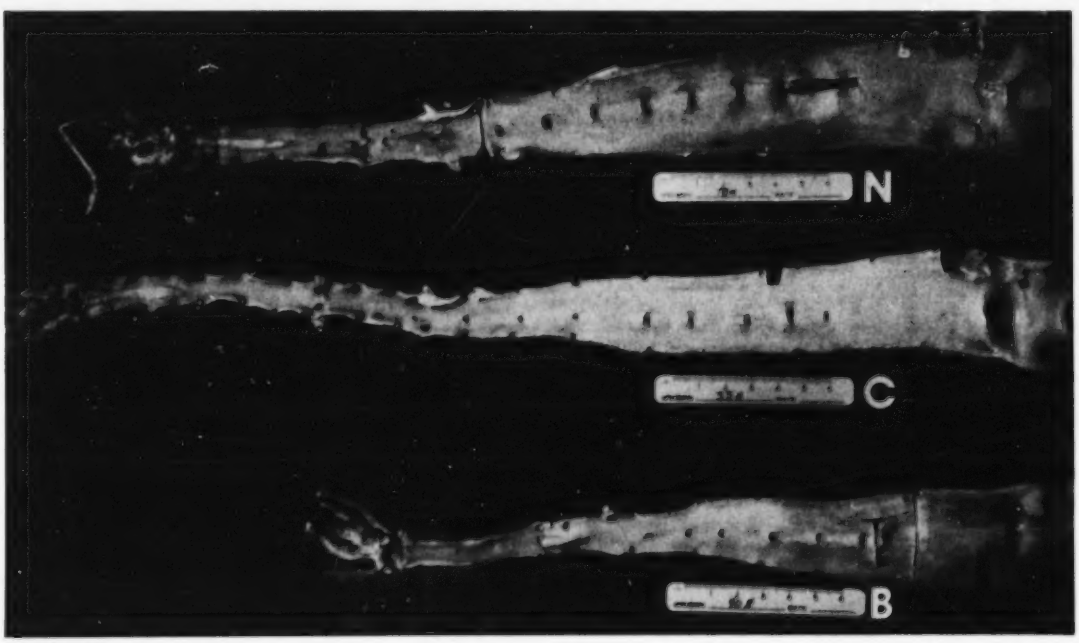

Figure 3—Aortas from normal (*N*), carrier (*C*), and bleeder (*B*) pigs showing sudanophilia and distal raised plaque. (With a photographic reduction of 7%)

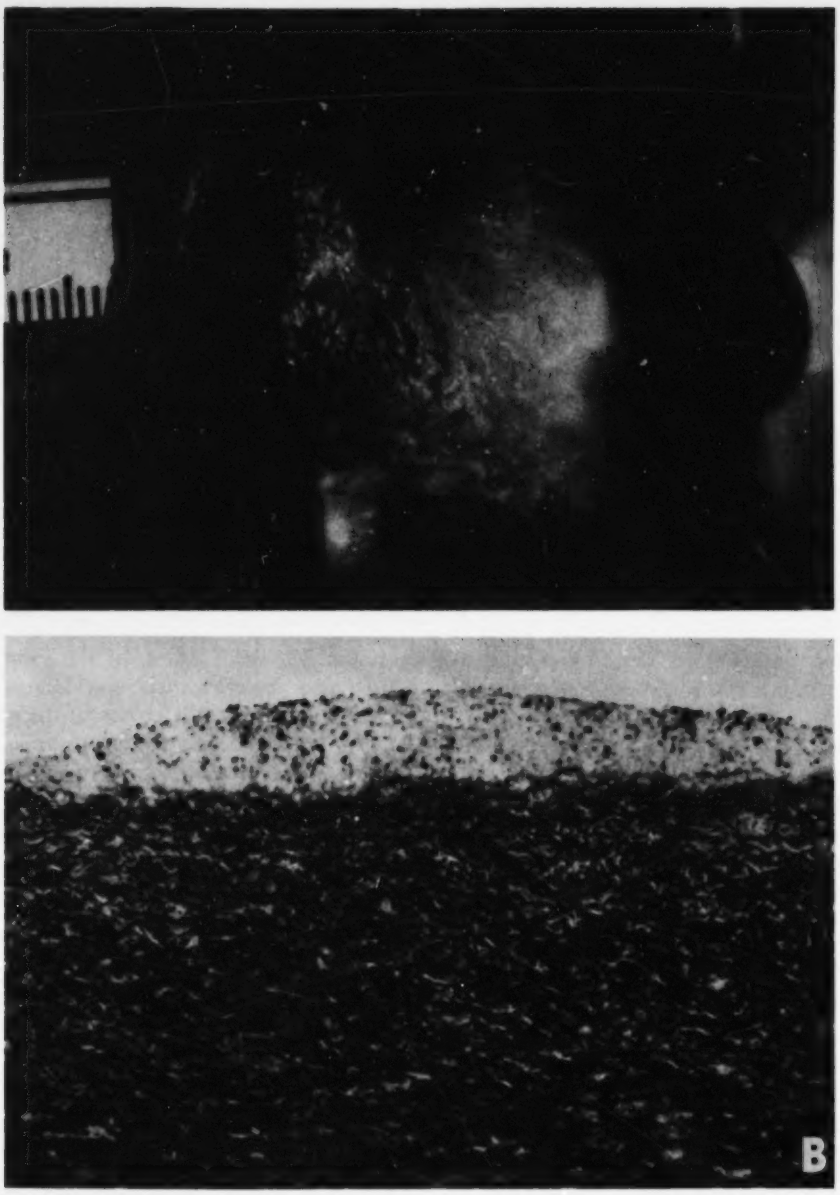

Figure 4—A raised plaque in the arch of the aorta of a normal pig. The corresponding light micrograph shows the lesion to be composed of intimal smooth muscle cells and intracellular and extracellular fat. (H&E, × 40)

by surface mapping because of the indistinct border between areas of lesion and normal vessel.

The third lesion on the aortas was a raised, linear plaque that was confined almost entirely to the distal aorta with frequent major involvement of the trifurcation (Figure 5). The microscopic appearance of the lesion was uniform, showing a central portion composed of foam cells covered by a cap of smooth muscle cells and fibrous tissue. These lesions were always well demarcated from the surrounding normal intima. The normal aortas contained an average of 3.88% involvement with this distal lesion with a standard deviation of 1.76. Bleeder aortas showed an average of 2.37% involvement with the lesion (SD ± 1.03). This difference is significant at the $P < 0.05$ level. The extent of involvement in carrier pigs was

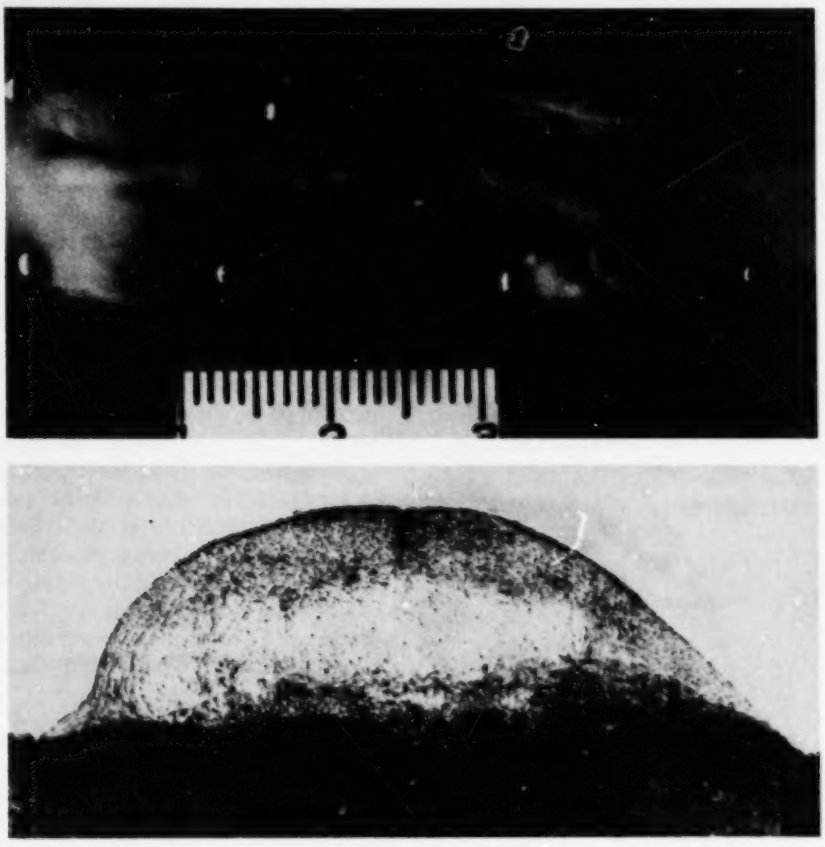

Figure 5—Raised fibrous plaque from the distal aorta of a bleeder pig. The light micrograph shows the plaque to consist of a core containing predominantly lipid with a cap of smooth muscle cells and fibrous matrix. (H&E, ×25)

2.83% (SD ± 1.40), which was not significantly different from either the value in the normals or that in the bleeders.

Myocardial Infarction and Sudden Death

Two experimental pigs, 1 carrier and 1 normal, developed myocardial infarction. One of these pigs, a carrier, died suddenly (Figure 6). Coronary thrombosis was not demonstrated in either pig.

In each case of myocardial infarction, the scarred area was composed of organized fibrous tissue with occasional areas of viable myocardium. The myocardium, in turn, contained numerous atypical myocytes with large, eccentric nuclei. There were scattered histiocytes in these areas, but no neutrophils were present. Both pigs with myocardial infarction showed development of electrocardiographic Q waves indicative of transmural myocardial infarction during the postballoon period.

Discussion

These experiments were designed to test the effects of balloon catheter injury on the development of coronary atherosclerosis in pigs with von Willebrand's disease. The results showed that balloon-injured vessels developed more severe atherosclerotic disease than did the coronary arteries that were not ballooned. However, the degree of lesion development in coronary arteries, both ballooned and nonballooned, was similar in the two von Willebrand phenotypic groups and the normal pigs. On the other hand, the disease in the aortas of the homozygous bleeder pigs tended to be slightly less extensive than that in the aortas of the normal pigs. The lesser degree of aortic disease in bleeder pigs as compared with normals is consistent with previous observations by Fuster et al.[12] It is interesting to note that the duration of the atherogenic diet in these experimental pigs was 4 months, while the term of the diet for the pigs studied by Fuster et al was 6 months. In spite of

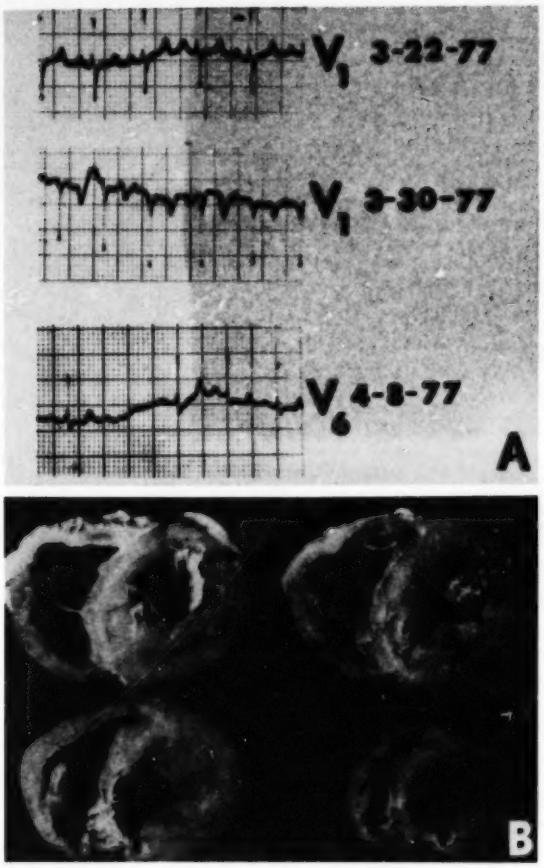

Figure 6A—Segments of serial electrocardiograms from a carrier pig. The heart from this pig is shown below. The tracing marked 3/22/77 was done 2 months after the ballooning and shows normal R waves in Lead V1. The tracing done 8 days later (3/30/77) shows new Q waves in Lead V1. The tracing from 4/8/77 showed several episodes of Mobitz type II heart block, a condition that might be related to the extensive anterioseptal infarction and the sudden death that occurred in this animal on 4/12/77. The left anterior descending coronary artery from this pig was partially occluded with an atherosclerotic plaque. No thrombus was present. The circumflex coronary artery was also partially occluded by plaque. The left anterior descending and circumflex arteries were both balloon-injured. **B**—Segments of ventricular myocardium showing the site of a healing infarction with scarring and thinning in the area of the left ventricle supplied by the left anterior descending coronary artery.

this comparatively shorter experimental period, our bleeder pigs showed considerable aortic lesion development. These observations thus demonstrate that the influence of the von Willebrand factor is not essential for lesion development and progression in the diet-induced porcine model. Further, our data suggest that the magnitude of the influence that the von Willebrand factor has on the atherogenic process is not so much a function of the type and degree of vessel wall injury as it is a function of the arteries involved.

Hemodynamic factors have long been thought to influence the development of atherosclerosis; and hemodynamic differences between coronary arteries and aortas would appropriately be suspected as factors involved in the disparate development of atherosclerosis in these two groups of arteries. How might the effects of hemodynamic differences be brought out by the absence of von Willebrand factor? Interestingly, Baumgartner et al have shown that the absence of von Willebrand factor in the perfusate of a test chamber does not seem to affect platelet adherence to rabbit aortic subendothelium when shear forces at the fluid-vessel wall interface are low.[20] Shear forces at which the absence of von Willebrand factor could be shown to depress the platelet adherence exist in life in vessels that are smaller than either the aorta or coronary artery. According to this hypothesis, therefore, the von Willebrand factor should have little or no effect on platelet adherence to injured vascular endothelium in either porcine aortas or coronary arteries. In other words, the varying hemodynamic parameters that may exist in the coronary arteries and aortas may not influence the function of the von Willebrand factor in those vessels in normal pigs.

Pigs taking an atherogenic diet are not "normal," however. A major factor to be considered in these pigs is the presence of high concentrations of lipid in the plasma. In *in vitro* studies Stuart et al have shown that exposure of human platelets to high concentrations of lipid promotes increased arachidonic acid product release.[21] If platelet metabolism is altered in this manner *in vivo*, the alteration might well affect the influence of von Willebrand factor function on platelets and on the interaction of platelets with the walls of larger vessels.

The high concentration of plasma lipids in these experimental animals might also affect von Willebrand factor function in larger arteries by altering the normal blood flow patterns. Turitto et al have recently shown that the degree of platelet adherence to denuded endothelium in an *in vitro* system was directly related to the erythrocyte hematocrit of the perfusate.[22] The authors suggested that high concentrations of erythrocytes displaced platelets and brought about more platelet-vessel wall contact. It is conceivable that high concentrations of lipid might influence platelet adherence by a related mechanism and thereby magnify the role of the von Willebrand factor in the platelet-vessel wall interaction in large arteries.

Therefore, factors such as hyperlipidemia might alter the metabolism of platelets or the physical relationship between the platelets in flowing blood and

the vessel wall so that effects of von Willebrand factor can become manifest in large vessels. In such a situation, the differences in the dynamics of the flowing blood in aortas and coronary arteries might influence the manifestation of von Willebrand factor activity to cause a disparate degree of atherosclerosis in these two areas of the arterial system.

It is noteworthy that myocardial infarction occurred in two animals, a normal pig and a carrier. In neither case of infarction was coronary thrombosis demonstrated. Our methods do not allow us to rule out the possibility that previously existing thrombosis had resolved before the pigs died or were sacrificed. Similarly, there are too few animals in this sample to allow us to comment on the intriguing possibility that bleeder pigs with coronary atherosclerosis might be protected from developing infarction or suffering sudden death.

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